Impurity Identification by Peptide Sequencing using 2D-LC MS/MS

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Background

As peptides become increasingly more prevalent as therapeutic options, it has become essential to achieve greater separation and identify difficult to resolve impurities. 2D-LC offers much greater levels of resolution by allowing peaks in the 1st dimension to be further separated in the 2nd dimension. The following case study presents the analytical workflow used to identify an unknown peak, closely eluting in the tail of an API peak, using a 2D-LC system coupled to a Q-TOF mass spectrometer.

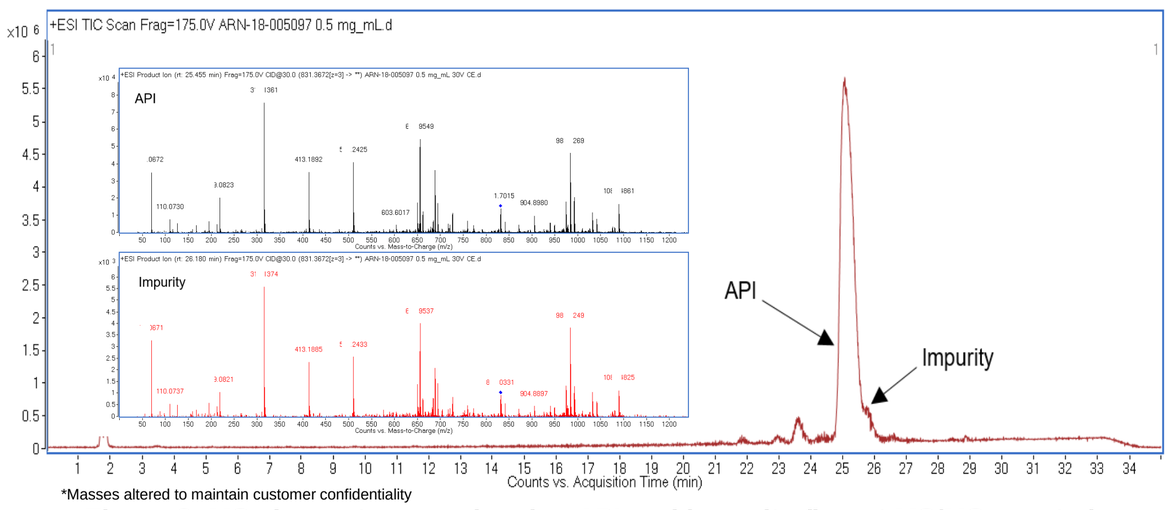
Analytical Equipment

Analysis was performed using an Agilent 1290 Infinity II 2D-LC system coupled with an Agilent Accurate Mass 6530 Q-TOF Mass Spectrometer (Figure 1). Agilent OpenLab ChemStation software was used to take a fraction (known as a heartcut) of the impurity and Agilent MassHunter software with BioConfirm was used for amino acid sequencing.

Figure 1: Agilent 1290 Infinity II 2D-LC (left) and Agilent Accurate Mass 6530 Q-TOF (right).

LC-MS and LC-MS/MS Analysis

LC-MS was initially used to try and determine the accurate mass of the API and the impurity in the tail of the API. The impurity was observed to have the same mass as the API. Fragmentation data was then generated by LC-MS/MS (Figure 2) to determine the identity of the impurity. The sequence map of the fragments present within the impurity matched with the API. However, during precursor selection, the software could not separately identify the peaks, so the level of contribution of API ions to the fragmentation profile of the impurity could not be assessed.



*Masses altered to maintain customer confidentiality

Figure 2: MS chromatogram showing API and impurity (insert MS/MS spectra).

2D Separation

Due to potential interference from the significantly more intense API spectra on the impurity spectra, it was necessary to achieve greater resolution. 1Dx1D was chosen as a 2D separation technique, which involves taking a heartcut from the 1st dimension and transferring it into the 2nd dimension, where the 1st dimension chromatographic conditions were replicated.

2D analysis enhances peak resolution, separating the compounds in the 2nd dimension by the same mechanism as the 1st dimension. As a result, the elution order of the API and impurity would remain the same but with increased impurity response relative to the API and better resolution, giving more confidence in the results obtained.

By increasing the injection volume and using 2D heartcut separation, greater resolution of the impurity peak was obtained (Figure 3). The impurity was observed to have a greater response than the API, due to the heartcut being taken form the most intense region of the impurity, which limited the amount of API present.

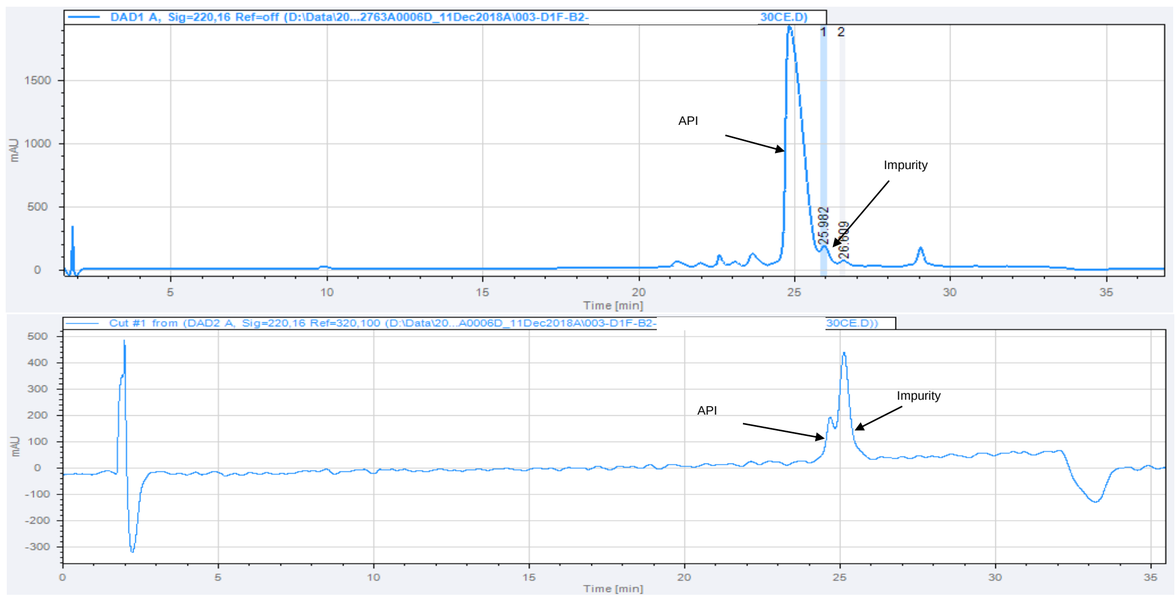


Figure 3: 2D 1st dimension (Top) and 2nd dimension (Bottom) UV Chromatograms.

2D-LC MS/MS Analysis

The increased resolution obtained (Figure 3) using 2D separation permitted MS/MS fragmentation profiles to be generated for both the API and impurity, with reduced concern for API interference. MassHunter was able to correctly identify the two peaks as separate compounds for precursor selection for MS/MS, which was not possible in 1D analysis.

BioConfirm Peptide Sequencing

The fragmentation data generated was processed using MassHunter's "Molecular Feature Extraction" algorithm. The peptide sequence was assigned against the theoretical peptide sequence using the MassHunter BioConfirm sequencing tool (Figure 4). Peptide sequencing of the fragments observed, indicated the impurity and API sequence profile aligned, which was consistent with expected results for a chiral isomer.

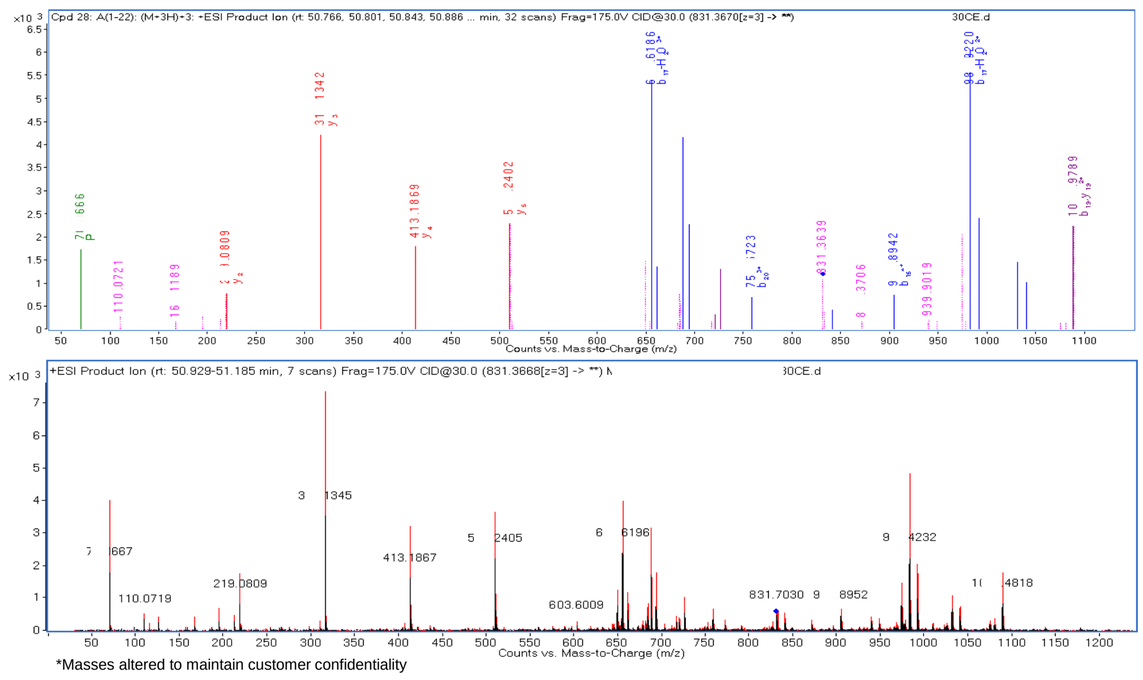


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Figure 4: Assigned API peptide sequence (top) and overlaid API vs impurity fragmentation profiles (bottom).

Conclusion

Uncertainty in the initial results arose due to incorrect precursor selection by the software, caused by poor resolution of the impurity peak. 2D separation provided greater resolution, which allowed the software to successfully select precursor ions from each peak. The fragmentation data obtained by 2D-LC MS/MS analysis confirmed that the sequence profiles were identical for both the API and the impurity, which was consistent with a chiral isomer of the API.